Exhibit E

Effect of setting pH on the properties of Mozzarella cheese made by direct acidification of whole milk standardized with dry milk protein concentrate

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Summary

The pH of milk at setting affects the properties of Mozzarella cheese. Milk protein concentrate (MPC) containing approximately 64% protein, 20% lactose and 2% calcium was used to standardize whole milk to a protein to fat ratio of ~1.3 for the manufacture of low-moisture part-skim (LMPS) Mozzarella cheese. Our objective was to compare the effect of setting pH (pH 5.6, 5.8 or 6.0) of whole milk standardized with MPC on LMPS Mozzarella cheese made by direct acidification. Standardized, pasteurized (72°C × 16 s) milk was divided into three lots A, B and C, and adjusted to pH 5.6 (A), 5.8 (B) and 6.0 (C), respectively, with 2% citric acid prior to setting (5 mL chymosin / 100 kg milk) and Mozzarella cheesemaking in triplicate trials. All cheeses were stored at 4°C for 35 days. Composition, yield, meltability, baking properties and hardness of the cheeses were determined by standard methods; primary proteolysis was assessed by urea-polyacrylamide gel electrophoresis and determination of water-soluble N contents of the cheeses. Significant differences ($p < 0.05$) in the % moisture (51.54 ± 2.09, 50.87 ± 2.32, 47.94 ± 1.85) and calcium contents (367.5 ± 1.2, 457.6 ± 4.2, 537.5 ± 2.1 mg/kg cheese) were found for cheeses A, B and C, respectively. No significant ($p > 0.05$) differences were observed in lactose, protein, fat, protein or fat recovery, and yield of cheeses. The % total solids recovered increased significantly with increase in setting pH of milk. Milk that was set at pH 5.6 gave the cheeses with best average meltability, least hardness, minimum browning while baking on pizza and highest levels of proteolysis.

Introduction

Mozzarella cheese constituted 32.7% of the total cheese produced in the U.S. in 2002 (USDA, 2003). About 70% of the mozzarella cheese produced in the U.S. is used in pizza manufacture. Mozzarella cheese is manufactured either by culture acidification method or direct acidification method. In direct acidification technique, cheese milk is acidified with food-grade acid or acidogen to pH (5.6-5.8) prior to cheesemaking. Acidification of milk results in partial loss of colloidal calcium from casein micelles into the milk serum and is subsequently loss in the whey. Loss of calcium from cheese improves meltability. Also pre-acidification of cheese milk leads to increased proteolysis in cheese because of increased retention of coagulant (Farkye, 1995) thereby, affecting meltability. The overall advantages of direct acidification include rapid and consistent make time, improved moisture retention, precise pH control and the elimination of culture expense and variability. Fife *et al.* (1996) and Merril *et al.* (1994) pre-acidified milk for the manufacture of low– and reduced–fat Mozzarella cheese to improve melting and stretching characteristics.

The effects setting pH of standardized milk (cream and skim milk) on LMPS Mozzarella cheese was studied by Metzger *et al.* (2000, 2001a, b) who found that pre-acidification to pH 5.8 with citric acid resulted in lower calcium in cheese than pre-acidification with acetic acid. Also, the chemical composition of cheese was not affected by pre-acidification pH of 5.8 or 6.0 except that calcium level was reduced at the lower pH.

The effects of fortifying milk with low-heat nonfat dry milk (NDM) on composition, proteolysis and properties of LMPS Mozzarella cheeses has been studied by Yun *et al.* (1998), who reported that fortification of milk with 3% NDM decreased proteolysis and increased firmness of cheeses during refrigerated storage. NDM fortification also resulted in increased browning, possibly due to the high level of lactose (~49%) in NDM. Milk protein concentrate (MPC) – manufactured by spray drying of retentate from ultrafiltered milk and containing a relatively high protein (64%), low lactose (~20%) and high calcium (~2%) contents compared to NDM – was used to standardize milk for the manufacture of reduced-fat cheese with enhanced yields and improved quality (Shakeel-Ur-Rehman *et al.*, 2003a, b). Shakeel-Ur-Rehman *et al.* (2003c) manufactured pizza cheese from standardized milk (whole milk plus MPC) by culture and direct acidification techniques. They found that cheese made from milk standardized with MPC by culture acidification technique had reduced meltability compared to that made by direct acidification. Also, they reported that pizza cheese made from milk that was acidified to pH 5.6 before setting was very soft. The reduction in the calcium content of cheese due to acidification of milk results in reduced cross-linking between caseins making the cheese soft (Soloroza and Bell, 1995). Hence, the present study was undertaken to determine the effect of setting pH of whole milk standardized with MPC to different pH values (5.6, 5.8 and 6.0) on the properties of LMPS Mozzarella cheese.

Materials and methods

Cheese manufacture

Raw milk (containing 3.8% fat, 3.6% protein and 4.7% lactose) and MPC (64% protein, 20% lactose, 5% moisture, 3.1% fat and 7.5% ash) were blended in a tank to give standardized cheese milk with a protein/fat ratio of ~ 1.3. MPC was obtained from Main Street Ingredients, La Crosse, WI. Standardized milk (~ 400 kg) was pasteurized at 72°C × 16 s using a universal pilot plant pasteurizer (PMS, Processing Machine and Supply Co, Philadelphia, PA) having capacity to process 1.9 L of milk per min and cooled to 4°C. The beginning and end of each standardized milk (~10 kg) exiting the HTST was discarded and 100 kg at 4°C was poured in each of the three vats A, B and C for cheesemaking.

The standardized milks in vats A, B and C were directly acidified to pH 5.6, 5.8 and 6.0 using ~9.24, 7.56 and 5.04 L, respectively, of 2% cold (3°C) citric acid. Citric acid was selected because it chelates calcium. After addition of citric acid, the milk was warmed to 31°C. Then, chymosin (Chymax™, Chr. Hansen's Lab., Milwaukee, WI) was added at the rate of 5 mL/100kg milk. Chymosin was diluted in distilled water in the ratio of 1:50 before adding to the vat. It took ~15 min for curd formation. The coagulum was cut with 1-cm horizontal and vertical curd wire knives. The cut curd was healed for 5 min and the curds were cooked to 36°C over 30 min followed by stirring for additional 5 min. The curd was allowed to sit in the whey for 30 min before drainage. Following whey drainage, the curds were stretched in hot water at ~82°C (180°F). The stretched curds were molded into approximately 2.7 kg loaves and placed in cold (3°C) brine (20% NaCl + 0.01% $CaCl_2$, pH 4.6) for 12 h. After brining, the cheese blocks were vacuum packaged in Cryovac™ (Sealed Air Corporation, Duncan, SC) bags and stored at 4°C for 35 days.

Yield

Actual cheese yield was determined by weighing the cheese obtained in each vat after removal from the brine and expressed as a percentage of the milk weight. Moisture-adjusted yields were calculated by theoretically adjusting the moisture content of cheese to 50%.

Fat, Protein and Total Solids Recovery in Cheese

Fat, protein and total solid recoveries were obtained by calculating the kg fat, protein or total solids in the cheese as a percentage of the kg fat, protein or total solids in milk from which cheese was made.

Compositional analyses

The fat contents of milk and cheese was analyzed by the Babcock method (Marshall, 1992). Total solids in the milk and whey, and moisture content of cheese were determined by the microwave oven method (CEM AVC 80 microwave oven, CEM Corporation, Matthew, NC) (Marshall, 1992). Protein (total N $\times$ 6.38) was determined by the Kjeldahl method (AOAC method 920.123; AOAC, 2000). Salt (NaCl) by determined by titrimetric method using the Corning 926 Chloride analyzer (Corning, Medfield, MA), and lactose by enzymatic method (R-Biopharm Inc., Marshall, MI). Calcium in the cheese was determined by AOAC method 991.25 (AOAC, 2000) by using a 3030B Atomic Absorption spectrophotometer (Perkin Elmer, Norwalk, CT).

Functional properties

Meltability of the cheeses was determined by a modified method of Olson and Price (1958) as described by Shakeel-Ur-Rehman *et al.* (2003c). A 15-g sample cut out of a block with a small circular cutter was placed in a cylindrical glass tube open on both ends. The end in which the cheese sample was placed was closed by a rubber cork. The rack was set at an inclined (45°) position in an incubator at 32°C for 1 h to temper the cheese. Then, the rack was set on the horizontal position at 110°C for 60 min. The meltability (cheese flow) was measured as the distance (cm) that the cheese had flowed from the original position after the tempering period. Baking property of the cheeses was determined by spreading shredded cheese (200 g) on commercial pizza dough (27.5 cm diameter) containing commercial pizza sauce (90 g) on the surface followed by baking in continuous pizza oven (Lincoln Impinger oven Model 1301, Wayne, IN) at 232°C for 3.25 min. The conveyor speed was 0.33 rpm. The number of blisters was counted on the entire surface of the pizza (27.5 cm diameter) and reported as number of blisters per cm. Cheese browning was empirically done by visualization of the surface of cheese on baked pizza.

Cheese hardness – force required to compress 50 or 70% height of a 15 g sample of the cheeses during storage period was done by a TATXT2 Texture analyzer (Texture Technologies Corporation, Scarsdale, NY) using method of Bhaskararacharya and Shah (2000).

Proteolysis

The cheeses were sampled after 7, 21 and 35 d of storage and frozen at (–20°C) until analyzed for proteolysis by urea-polyacrylamide gel electrophoresis (urea-PAGE) according to the method described by Farkye (1995) and by determining the Kjeldahl N content in the water-soluble fraction (WSF) of the cheeses. The WSF were prepared according to the method of Kuchroo and Fox (1982). The N content of the WSF was expressed as a percentage of the total N in cheese and reported as %WSN.

Statistical Analysis

All analytical measurements were done in duplicate for each of the three cheesemaking trials. Data was analyzed statistically by oneway ANOVA using Minitab statistical software package for Windows 98 (Minitab Inc, State College, PA).

Results and discussion

Composition of milk and cheese

The composition of cheese milk is presented in Table 1. Addition of citric acid did not affect the fat, protein not total solids contents of cheese milk. The composition of cheese is given in Tables 2. When the setting pH was decreased, the calcium content of the cheese was significantly lower ($p < 0.009$) and the moisture content significantly higher ($p < 0.021$). The reduction in the setting pH of milk promoted solubilization of micellar calcium phosphate (van Hooydonk et al., 1986) which is lost in the whey. The increase in moisture content of the cheese at lower setting pH is due to increased hydration of caseins. Sood et al. (1979) reported that caseins are more hydrated as the level of bound calcium decreases in milk. Similarly, Guinee et al. (2002) found that Mozzarella cheese made by direct acidification with lactic acid (setting pH of 5.6) had higher moisture content than that made with setting pH of 6.6.

Cheese Yield

The average actual yield of LMPS Mozzarella cheese was ~17.2% whereas the average yield for cheese adjusted to 50% moisture was 17.3%. Both actual and moisture-adjusted yields were not influenced by setting pH of milk (Table 2). Metzger *et al.* (2000) also reported that actual yields of LMPS Mozzarella cheese made from standardized milks that were set at pH 5.6 and pH 6.0 were not significantly different from control cheeses made from normal milk. Typical yield of LMPS Mozzarella cheese manufactured from part-skim milk is ~10% (Shakeel-Ur-Rehman *et al.*, 2003c) but higher in this study because of the high solids milk used for manufacture. Similar results were reported by Shakeel-Ur-Rehman *et al.* (2003c) for pizza cheese made from whole milk standardized with MPC.

Protein and fat recovery in cheese was not influenced by setting pH of the milk. However, the level of TS recovered in the cheese was significantly influenced by the setting pH of milk. Metzger *et al.* (2000), also found that actual fat and protein recoveries in LMPS Mozzarella cheese were not affected by the setting pH of milk (pH 5.6 or 6.0). The reduced recovery of total solids in the cheese at lower setting pH of milk could be due to loss of casein structure from solubilization of calcium, hence loss of minerals in the whey and during stretching of the curd.

Functional properties

The Mozzarella cheese made from standardized milk that was set at pH 5.6 or 5.8 had significantly ($p < 0.001$) higher meltability (Figure 1) than cheese made from milk acidified to a setting pH of 6.0 during the first 21 d of storage. However, at 35 d of storage, the cheese made from milk that was set at pH 5.8 had highest meltability ($p < 0.001$). The cheese made from milk, which was pre-acidified to pH 6.0 had lowest meltablity during the entire storage period. The cheese made from milk that was set at to pH 5.6 gave least browning on pizza but cheese made from milk that was set at 5.8 produced highest numbers of blister per cm^2.

Proteolysis

The WSN (as % of total N) contents were significantly ($p < 0.001$) higher in the cheese made from milk set at pH 5.6 or 5.8 than in those made from milk set at pH 6.0 (Table 6). Urea-PAGE electrophoretograms of the cheeses (Figure 2) showed higher levels of degradation of α_{s1}-casein in cheese made from milk with a setting of pH 5.6 and 5.8 compared to that in cheese made from milk with a setting pH 6.0. Reducing setting pH of milk increases the retention of coagulant (Farkye, 1995) in cheese, thereby increasing intensity of proteolysis. Farkye *et al.* (1991) reported that considerable proteolysis in culture acidified Mozzarella cheese (pH 5.2) occurs during storage and that soluble N increases from 4% of total N to 10% after 14 d.

Rheology

The force (g) required to compress cheese samples to 50% or 70% height is given in Table 5. Generally, the cheeses were softer with the decrease in setting pH of the milk. The increase in softness of cheeses with the reduced setting pH of milk is due to loss of micellar calcium in whey. The loss of micellar calcium results in weakening of casein matrix allowing more moisture to be incorporated in the matrix, thus making the cheese softer. The force required to reduce cheese samples to 70% of original height (a measure of cheese hardness) showed no significant difference ($p > 0.01$) among treatments, probably force to compress the first 50% height get maximum resistance due to composition of cheese.

Conclusions

Mozzarella cheese (7 day or 21 day-old) with best meltablity i.e. cheese flow when incubated samples were incubated at 110 °C was obtained when the standardized milk was pre-acidified to pH 5.6 compared to those cheeses from milks that were pre-acidified to pH 5.8 or 6.0. The cheese made from milk with a setting pH of 5.6 also had least browning when spread on Pizza. Cheese made from milk with a setting pH of 5.6 had the lowest calcium content resulting in cheese with a soft texture. It is concluded that setting the pH of MPC fortified milk to 5.8 may be good for the manufacture of directly acidified Mozzarella cheese that is to be held for more than 21 d before use in pizza baking because its meltability at 35 d was better than pH 5.6 cheese and has more calcium than the cheese made from milk with a 5.6 setting pH.

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Table 1. Composition* (mean ± standard deviation) of standardized milk (made by mixing whole milk and milk protein concentrate) for Mozzarella cheese manufacture.

Milk pH	% Fat	% Protein	% Casein	% Total solids
5.6	3.75 ± 0.04	4.76 ± 0.93	3.61 ± 0.11	14.65 ± 0.11
5.8	3.75 ± 0.04	4.77 ± 0.08	3.62 ± 0.11	14.69 ± 0.13
6.0	3.75 ± 0.04	4.76 ± 0.07	3.63 ± 0.10	14.60 ± 0.08
p-value	1.00	0.976	0.961	0.873

* Mean of three trials analyzed in duplicate.

Table 2. Percent* yields and fat, protein and total solid recoveries during Mozzarella cheese manufacture.

Milk pH	Yield		Component recovery		
	Actual	Adjusted[1]	Fat (%)	Protein (%)	Total solids (%)[2]
5.6	17.02 ± 0.12	16.51 ± 0.12	85.38 ± 9.29	72.16 ± 2.20	55.56 ± 2.03
5.8	17.86 ± 0.97	17.55 ± 0.97	94.42 ± 7.50	77.13 ± 5.40	59.79 ± 5.33
6.0	16.65 ± 0.67	18.02 ± 0.67	95.24 ± 3.67	76.44 ± 2.25	61.11 ± 0.55
p-value	0.13	0.13	0.06	0.07	0.03

* Mean of three trials analyzed in duplicate.

[1] Adjusted to 50% moisture

[2] Computed after subtracting moisture contents from the cheeses.

Table 3. Composition (mean ± standardization) * of 7 day-old Mozzarella cheeses.

Milk pH	Moisture (%)	Fat (%)	Protein (%)	Salt (%)	Ca (mg/100g)	Lactose (%)
5.6	51.54 ±2.09	18.83 ± 2.20	20.55 ± 0.75	2.29 ± 0.31	367.5 ± 1.83	0.75 ± 0.0)
5.8	50.87 ± 2.32	19.83 ± 0.93	20.64 ± 1.21	2.05 ± 0.42	475.5 ± 4.24	0.86 ± 0.03
6.0	47.94 ± 1.85	20.66 ± 0.51	21.40 ± 0.36	2.19 ± 0.16	537.5 ± 0.20	0.79 ± 0.12
p-value	0.021	0.113	0.202	0.445	0.009	0.10

* Mean of three trials analyzed in duplicate.

Table 4. Baking properties in Mozzarella cheese on pizza cooked in oven at 232°C for 5 min.

Milk setting pH	Browning	Number of blisters / cm
5.6	+	2
5.8	++	4
6.0	+++	none

[+] least or no browning

[+++]some browning

[+++] too much or unacceptable level of browning.

All values are means of three trials.

Table 5. Water-soluble N (as % total N) during storage of pizza cheese sample made from whole milk and milk protein concentrate.

Milk setting pH	7 d	21 d	35 d
5.6	22.57 ± 8.02	18.37 ± 3.61	32.54 ± 8.48
5.8	13.87 ± 3.53	15.65 ± 2.29	24.02 ± 9.11
6.0	7.38 ± 0.45	7.7 ± 0.61	8.54 ± 1.23
p-value	0.001	0.001	0.01

* Mean of three trials.

Table 6. Force (g) needed to compress Mozzarella cheese samples of various ages to 50 or 70% original height.

	Force to compress cheese to 50% height			Force to compress cheese to 70% height		
Milk pH	7 d	21 d	35 d	7 d	21 d	35 d
5.6	3572 ± 542	2527 ± 1049	2278 ± 742	11073 ± 7175	11457 ± 4880	10975 ± 6291
5.8	3379 ± 451	3022 ± 1264	2824 ± 928	8564 ± 1354	7891 ± 2485	7036 ± 2266
6.0	5038 ± 1231	5472 ± 1224	4307 ± 1543	10285 ± 2816	10837 ± 4268	9125 ± 2180
p-value	0.006	0.001	0.019	0.134	0.288	0.274

Figure legends

Figure 1: Meltability* (cm) of Mozzarella cheese of various ages heated at 110°C for 60 min.

Figure 2: Urea-polyacrylamide gel electrophoresis of Mozzarella cheeses. Lane 1 is sodium caseinate, lanes 2, 3, 4 are 7 day-old cheeses made from milk acidified to pH 5.6, 5.8 or 6.0, respectively, before renneting. Lanes 5, 6, 7 represent cheeses after 27 d storage, and 8, 9, 10 are for cheeses after 35 d storage.


12
10
8
6
4
2
0
Meltablity (cms)
7
21
35
Age of cheese (days)
pH 5.6
pH 5.8
pH6.0

Figure 1.

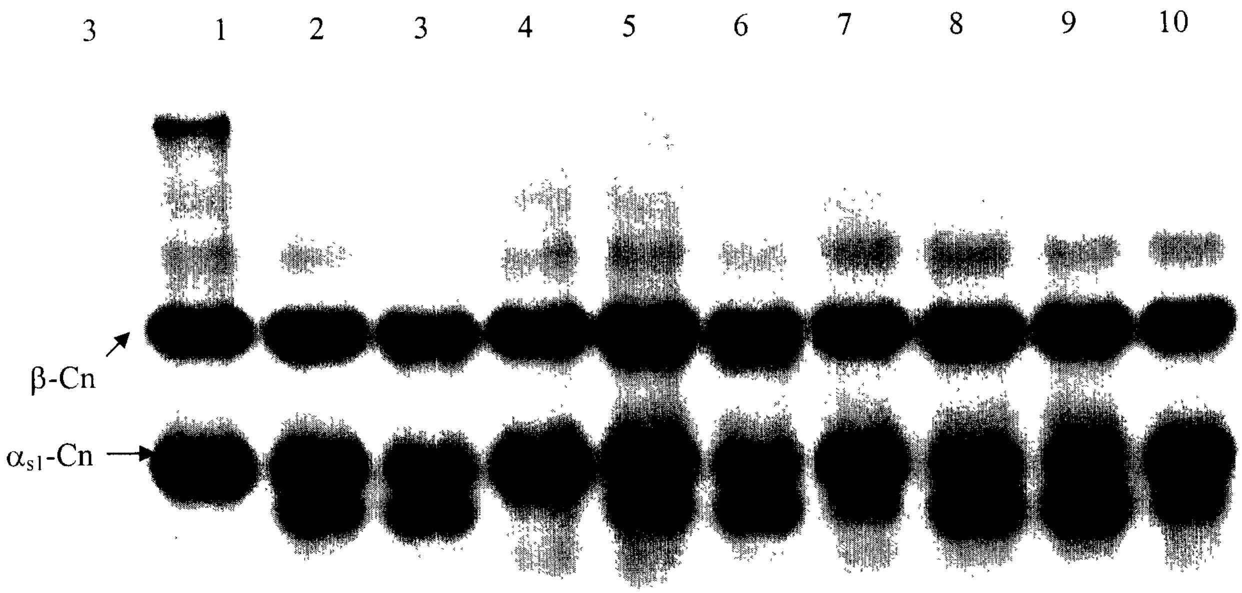

Figure 2.